Molecular and Cellular Endocrinology ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

The androgen receptor has no direct antiresorptive actions in mouse osteoclasts

Mieke Sinnesael [a,1], Ferran Jardi [a,1], Ludo Deboel [a], Michaël R. Laurent [b,c], Vanessa Dubois [b], Jeffrey D. Zajac [d], Rachel A. Davey [d], Geert Carmeliet [a], Frank Claessens [b], Dirk Vanderschueren [a,*]

[a] Clinical and Experimental Endocrinology, Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium
[b] Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium
[c] Gerontology and Geriatrics, Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium
[d] Department of Medicine, Austin Health, University of Melbourne, Heidelberg, Victoria, Australia

ARTICLE INFO

Article history:
Received 13 January 2015
Received in revised form 29 April 2015
Accepted 29 April 2015
Available online

Keywords:
Androgen receptor
Androgens
Bone marrow stromal cell
Bone resorption
Osteoblast
Osteoclast

ABSTRACT

Androgen deficiency or androgen receptor knockout (ARKO) causes high-turnover osteopenia, but the target cells for this effect remain unclear. To examine whether AR in osteoclasts directly suppresses bone resorption, we crossed AR-floxed with cathepsin K-Cre mice. Osteoclast-specific ARKO (ocl-ARKO) mice showed no changes neither in osteoclast surface nor in bone microarchitecture nor in the response to orchidectomy and androgen replacement, indicating that the AR in osteoclasts is not critical for bone maintenance. In line with the lack of a bone phenotype, the levels of AR were very low in osteoclast-enriched cultures derived from bone marrow (BM) and undetectable in osteoclasts generated from spleen precursors. Since tibiae of ubiquitous ARKO mice displayed increased osteoclast counts, the role of AR was further explored using cell cultures from these animals. Osteoclast generation and activity *in vitro* were similar between ARKO and wildtype control (WT) mice. In co-culture experiments, BM stromal cells (BMSCs) were essential for the suppressive action of AR on osteoclastogenesis and osteoclast activity. Stimulation with $1,25(OH)_2$ vitamin D_3 increased *Rankl* and decreased *Tnfsf11* (osteoprotegerin, *Opg*) gene expression in BMSCs more than in osteoblasts. This increase in the *Rankl*/*Opg* ratio following $1,25(OH)_2D_3$ stimulation was lower, not higher, in ARKO mice. *Runx2* expression in BMSCs was however higher in ARKO *vs.* WT, suggesting that ARKO mice may more readily commit osteoprogenitor cells to osteoblastogenesis. In conclusion, the AR does not seem to suppress bone resorption through direct actions in osteoclasts. BMSCs may however represent an alternative AR target in the BM milieu.

1. Introduction

Osteoporosis in men is less common than in women but still represents an important burden for public health (Khosla et al., 2008). Greater cortical bone expansion during peak bone mass acquisition is the main reason why the skeleton of men is stronger compared to women (Seeman, 2003). Androgens such as testosterone (T) stimulate periosteal bone formation through the androgen receptor (AR), whereas estrogens such as 17β-oestradiol (E_2) via estrogen receptor alpha (ERα) have an inhibitory effect on cortical bone expansion (Vanderschueren et al., 2014). Because in aging, men maintain their sex steroid levels better than women, they do not experience the accelerated phase of bone loss observed after menopause in women. However a slow decline in the levels of bioavailable androgens and therefore also in E_2, since androgens are the substrate for the aromatase enzyme and production of estrogens, could contribute to osteoporosis in aging men (Laurent et al., 2015). Although non-aromatizable androgens can prevent bone resorption in rodent models, it remains controversial whether this is also the case in humans. Several experimental studies (Falahati-Nini et al., 2000; Idan et al., 2010; Laurent et al., 2015) have demonstrated that androgens were unable to prevent bone resorption in the face of estrogen deficiency and although they may selectively influence bone formation (Falahati-Nini et al., 2000), this has not been shown to translate into preservation of bone mass in aging men.

Bone maintenance is determined by osteoclastic bone resorption and osteoblastic bone formation. Resorption and formation are coupled processes; indeed, osteoclasts release matrix-embedded factors and secrete the so-called clastokines which stimulate

* Corresponding author. Clinical and Experimental Endocrinology, KU Leuven, Herestraat 49 PO box 902, B-3000 Leuven, Belgium. Tel.: +32 (0)16 346987; fax: +32 (0)16 346989.
E-mail address: dirk.vanderschueren@uzleuven.be (D. Vanderschueren).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.mce.2015.04.030
0303-7207/

osteoblasts, while osteoblasts and other cells release factors such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) which modulate osteoclast generation and activity (Sims and Martin, 2014). In mouse models, androgens via the AR restrain bone turnover by inhibiting both osteoclastogenesis and osteoblastogenesis (Bellido et al., 1995; Kawano et al., 2003; Venken et al., 2006). However, whether osteoblasts, osteocytes, osteoclasts or other cells constitute the main targets for AR actions in restraining bone turnover remains unclear (Manolagas et al., 2013; Vanderschueren et al., 2014).

Osteoclast generation, activity and lifespan critically depend on M-CSF and RANKL secretion by osteoblasts, osteocytes and other cells in the bone marrow (BM) milieu (Arai et al., 1999; Bellido et al., 1995; Xiong et al., 2011). *Rankl* expression has been found upregulated in osteoblasts of ARKO mice, suggesting that the AR in osteoblasts is critical for the suppressive effect of androgens on osteoclast generation and activity (Kawano et al., 2003). Several animal studies have suggested that the AR might be expressed in osteoclasts and exert effects on these cells *in vitro* (Huber et al., 2001; Mizuno et al., 1994; Pederson et al., 1999; van der Eerden et al., 2002), although it remains controversial whether the AR inhibits osteoclasts directly. Conditional knockout models have demonstrated that ERα in osteoclasts mediates bone resorption in female but not in male mice (Martin-Millan et al., 2010; Nakamura et al., 2007). Similarly, a recent study in an osteoclast-specific deletion model for the AR has shown that the anti-resorptive effects of androgens are not derived from their direct action on these cells (Ucer et al., 2015). However, these new data contradict the results from the only previous study until now which had investigated the ablation of AR in osteoclasts *in vivo* (Nakamura et al., 2004). Therefore, further studies are required as to clarify the androgenic action in osteoclasts. Osteoblasts derive from the mesenchymal lineage via BM stromal cells (BMSCs), which commit to the osteoblast lineage once RUNX2 is expressed (Komori, 2011). Cre-mediated deletion of the AR in mature osteoblasts using the Col1a1 (Notini et al., 2007) or the osteocalcin (Chiang et al., 2009; Määttä et al., 2013) promoter showed respectively no increased osteoclast surface and increased osteoclast numbers/surface. Recently, we have shown using Dmp1-Cre mice that loss of AR in mature osteoblasts and osteocytes resulted in similar, moderate trabecular bone loss (Sinnesael et al., 2012). Although we could not demonstrate increased bone resorption in this model, the effect on trabecular number and the lack of difference in anabolic response to a mechanical loading stimulus suggests that also in this model, a slow, age-accumulating increase in bone resorption is responsible for the observed trabecular bone losses. These different models suggest a direct role of AR across the entire osteoblast lineage, although the data do not fully explain the suppressive effects of androgens on bone resorption (Imai et al., 2013).

The main goal of this study was to investigate to what extent the effects of androgens on osteoclasts are direct through the AR. For this purpose, we generated a specific knockout model in which the AR is ablated in mature osteoclasts. In addition, we compared the generation and activity of osteoclasts in primary cultures from BM of our global ARKO and wildtype control (WT) mice. Finally, we co-cultured osteoblasts from ARKO or WT mice with BM from ARKO or WT in order to explore potential alternative mechanisms explaining the anti-resorptive effects of the AR.

2. Materials and methods

2.1. Animal care

All transgenic mice had a >95% C57BL/6J genetic background. Mice were group-housed under conventional conditions: 12-h light/dark cycle, standard diet (1% calcium, 0.76% phosphate), and water *ad libitum* in standard cages. The Animal Ethical Committee of the KU Leuven approved all procedures (P143/2011).

2.2. Generation of global ARKO and osteoclast-specific ARKO (ocl-ARKO) mice

Global ARKO mice have been previously generated by crossing mice with an ubiquitous Cre to mice with a floxed second exon of the AR (De Gendt et al., 2004). Mice with an osteoclast-selective knockout of the AR (ocl-ARKO) were generated by crossing female mice heterozygous for a floxed exon 2 AR allele ($AR^{(ex2)}flox^{+/-}$) (De Gendt et al., 2004) with male mice heterozygously carrying a cyclization recombinase of which the expression is controlled by the cathepsin K promoter (Ctsk-Cre, kindly provided by Prof. R. Davey) (Chiu et al., 2004; Turner et al., 2011). Male offspring were weaned at 3 weeks of age and genotyped by PCR as described previously (De Gendt et al., 2004). The presence of the Ctsk-Cre transgene was determined on genomic DNA via PCR with forward primer: 5′-GCGGCATGGTGCAAGTTGAAT-3′ and reverse primer: 5′-ACCCCCAGGCTAAGTGCCTT-3′ resulting in a 534 bp fragment.

2.3. Orchidectomy and replacement

At the age of 12 weeks ocl-ARKO and control (ARfl/Y; Ctsk-Cre–/–) male mice were either sham-operated (sham), orchidectomized (ORX) or ORX and immediately given a silastic tube with testosterone (Serva, Heidelberg, Germany) replacement (ORX+T) using subcutaneous silastic implants (Silclear Tubing, Degania Silicone, Jordan Valley, Israel) in the cervical region (Vanderschueren et al., 2000). After 4 weeks animals were euthanized and efficacy of ORX and T replacement was verified by measurement of seminal vesicle wet weights. Femurs were collected and analyzed with μCT.

2.4. μCT analysis

μCT analysis was done *ex vivo* on the left femur by using a Skyscan 1172 scanner (Brüker, Kontich, Belgium) as described (Sinnesael et al., 2012). Cortical bone was analyzed starting at a distance of 2.75 mm from the growth plate and extending 0.5 mm distally (100 slices, 5 μm voxel size) to determine cortical area (Ct.Ar, mm^2), cortical thickness (Ct.Th, mm), periosteal bone perimeter (PsPm, mm), endocortical bone perimeter (EcPm, mm) and medullar area (Me.Ar, mm). The parameters evaluated in the trabecular region of the distal femur, commencing at a distance of 0.5 mm from the growth plate and extending a further 1.5 mm proximally, included bone volume/tissue volume (BV/TV,%), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm^{-1}) and trabecular separation (Tb.Sp, mm) (Callewaert et al., 2009).

2.5. Bone histomorphometry

Right tibias and femurs were prepared and stained with tartrate-resistant acid phosphatase (TRAP) to visualize osteoclasts as described (Sinnesael et al., 2012). Histomorphometric analysis was done by using a Zeiss Axiovert microscope and an Axiovision (v6.1.0) image analysis system (Daci et al., 2000; Masuyama et al., 2006). Osteoclast surface (Oc.S) was determined as % of bone surface (BS).

2.6. Primary bone marrow stromal cell and osteoclast-enriched cultures

In vitro osteoclast generation was assessed first by culturing BM-derived hematopoietic cells in the presence of exogenous M-CSF (R&D Systems, Abingdon, UK) and RANKL (Peprotech, Rocky Hill,

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

NJ, USA) as described (Daci et al., 2000; Verlinden et al., 2013). Briefly, BM cells were isolated from the long bones of hindlimbs of 8–10 week old global ARKO and WT mice and centrifuged on Ficoll–Paque gradient (Stem Cell Technologies, Grenoble, France) to isolate mononuclear cells. The BM cells were plated overnight in α-MEM supplemented with 10% FBS and containing 10 ng/mL M-CSF.

For osteoclast-enriched cultures, the non-adherent cells were harvested the next day and plated at 1.5×10^6 cells/well in a 48 well dish in α-MEM supplemented with 10% FBS and containing M-CSF (20 ng/mL) and RANKL (100 ng/mL). The medium was replaced every 3–4 days. After 7 days of culture, TRAP staining was performed after a 10 min fixation of cells with 4% paraformaldehyde and permeabilization for 5 min with 0.1% Triton X-100 (Sigma-Aldrich). Cells were subsequently incubated at room temperature in 0.1 M sodium acetate containing naphtol AS-MX phosphate and Fast Red violet LB salt (Sigma-Aldrich, Bornem, Belgium), in the presence of 1% dimethylformamide (Merck, Darmstadt, Germany). Cells stained positively and containing 3 or more nuclei were considered osteoclasts and counted on digital images with ImageJ software.

For BMSC cultures, the adherent cells were plated in a 6 well dish at 1.5×10^6 cells/mL in α-MEM medium supplemented with 10% FBS in the presence or absence of 10^{-8} M 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Sigma-Aldrich). Medium was replaced every 3 days. After 14 days of culture, the cells were used for RNA isolation.

2.7. Generation of osteoclasts from spleen precursors

Mature osteoclasts of WT and global ARKO mice were also obtained from spleen precursors by utilizing soluble forms of M-CSF and RANKL (Bradley and Oursle, 2008). Briefly, after centrifuging on Ficoll–Paque, spleen cells were cultured at 10^6 cells/mL in α-MEM supplemented with 10% FBS and containing M-CSF (20 ng/mL) and RANKL (100 ng/mL). The medium was replaced every 3–4 days. RNA from differentiated osteoclasts was extracted on day 7.

2.8. Co-culture of calvarial osteoblasts and hematopoietic precursor cells

Osteoblasts were isolated from calvariae of neonatal WT and global ARKO mice. Calvariae were sequentially digested with 0.1% collagenase A and 0.2% dispase (Sigma-Aldrich) as described previously (Daci et al., 2000). Cells released during digestion cycles 2–6 were pooled and used in experiments. BM cells were collected from the femurs of 8–10 week old control and global ARKO mice as described earlier. TRAP-positive multinucleated osteoclasts were generated by co-culturing calvarial osteoblasts (2×10^4 cells/well in a 48-well dish) and BM cells (2×10^5 cells/well in a 48-well) in α-MEM medium supplemented with 10% FBS and in the presence of 10^{-8} M $1,25(OH)_2D_3$. For osteoclast quantification, the average number of TRAP-positive cells with more than three nuclei X20 field was obtained at 8 days of culture.

2.9. Matrix dissolution assay

Osteoclasts were formed following the procedure of "Primary osteoclast enriched cultures" or "Co-culture of calvarial osteoblasts and hematopoietic precursor cells" directly on Osteo Assay Surface wells (Corning, Amsterdam, The Netherlands), which contains a matrix that osteoclasts can resorb. On day 7 the plate was stained for TRAP or bleached to visualize the resorbed surface area. The percentage of resorbed surface was assessed with ImageJ on digital images captured with an LSM 510 Axiovert 100M microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

2.10. RNA isolation, cDNA synthesis, and Q-PCR

Tissue samples (brain, adipose tissue, heart, liver, quadriceps, testis) as well as tibia, BM, osteoclast-enriched cells, hematopoietic cells, stromal cells and calvarial osteoblasts were obtained at 12 weeks of age and snap-frozen in liquid nitrogen. cDNA was synthesized from DNaseI-treated total RNA (RNeasy Kit, Qiagen, Chatsworth, CA, USA) by using superscript II $RNaseH^-$ reverse transcriptase and random hexamer primers (Invitrogen, Ghent, Belgium). The following primers and probes were used for the androgen receptor (AR): forward primer: 5′-GACATGCGTTTGGACAGTACCA-3′; reverse primer: 5′-TGACAGCCAGAAGCTTCATCTC-3′; probe: 5′-CCCAGAAGACCTGCCTGATCTGTG-3′ (amplicon = 82 bp); receptor activator of nuclear factor kappa-B ligand (RANKL): forward primer: 5′-CATTTGCACACCTCACCATCA-3′; reverse primer: 5′-TTGCTTAA CGTCATGTTAGAGATCTTG-3′; probe: 5′-TCGGGTTCCCATAAAGTC ACTCTGTCCTCTT-3′ (amplicon = 118 bp); osteoprotegerin (OPG): forward primer: 5′-GAAGGGCGTTACCTGGAGATC-3′; reverse primer: 5′-CTGAATTAGCAGGAGGCCAAAT-3′; probe: 5′-TCACCTGAGAAG AACCCATCTGGACATTTT-3′ (amplicon = 201 bp); runt-related transcription factor 2 (RUNX2): forward primer: 5′-TACCAGCCAC CGAGACCAA-3′; reverse primer: 5′-AGAGGCTGTTTGACGCCATAG-3′; probe: 5′-CTTGTGCCCTCTGTTGTAAATACTGCTTGCA-3′ (amplicon = 94 bp). Expression of the main osteoclastogenic cytokines, namely tumor necrosis factor alpha (TNF-α) and interleukin 1 and 6 (IL-1α, IL-1β and IL-6), was determined by using predesigned TaqMan® Assays (TNF-α; Mm00443258_m1, IL-1α; Mm00439620_m1, IL-1β; Mm00434228_m1 and IL-6; Mm00446190_m1). For quantification of gene expression, the ABI Prism 7500 sequence detector PCR detection system (Applied Biosystems, Ghent, Belgium) was used with a two-step RT-quantitative PCR protocol. The relative expression levels of the target genes were calculated as a ratio to the hypoxanthine–guanine phosphoribosyl transferase (HPRT) gene with the following primers: forward primer: 5′-TTATCAGACTGAAGAGCTACTGTAATGATC-3′; reverse primer: 5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′; probe: 5′-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3′ (amplicon = 127 bp).

2.11. Genomic DNA isolation of osteoclast enriched cells

Genomic DNA was isolated from osteoclast-enriched cells (see primary osteoclast enriched culture) with Qiagen blood & cell culture DNA minikit (Qiagen Benelux B.V., KJ Venlo, The Netherlands). The recombination of the AR gene was detected by PCR, as described previously (De Gendt et al., 2004).

2.12. Osteocalcin and TRAPCP5b assays

Serum osteocalcin and tartrate-resistance acid phosphatase (TRACP5b) were measured by an in-house radioimmunoassay (RIA) (CV intra-assay <5.9%; CV interassay <5.2%) (Verhaeghe et al., 1989) and by a MouseTRAP™ Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (ids, Elitech Benelux, Belgium) (CV intra-assay <7%; CV interassay <8%), respectively.

2.13. Statistical analysis

Statistical analysis was performed using NCSS software (NCSS, Kaysville, UT, USA). Student's *t* test and one-way ANOVA followed by Fisher's least significant differences multiple comparison test were performed to assess significance of differences between two or more groups. Data are represented as means ± SEM, and a *p*-value of <0.05 was considered significant.

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

3. Results

3.1. Generation and characterization of osteoclast-specific ARKO (ocl-ARKO) mice

To gain further insights regarding the possible target cell of AR-mediated androgen action on bone, the AR gene in mature osteoclasts was disrupted. In an initial experiment the seminal vesicle weight/body weight, femur length and bone parameters with µCT of 12-week-old WT ($AR^{WT/Y}$; Ctsk-$Cre^{-/-}$), hemizygous floxed ($AR^{fl/Y}$; Ctsk-$Cre^{-/-}$), hemizygous Ctsk-Cre ($AR^{WT/Y}$; Ctsk-$Cre^{+/-}$) and ocl-ARKO ($AR^{fl/y}$; Ctsk-$Cre^{+/-}$) were assessed (Supplementary Table S1). Because there were no differences in the bone parameters between the 3 control groups, we used flox mice ($AR^{fl/Y}$; Ctsk-$Cre^{-/-}$) as controls in our further experiments.

To further characterize this new mouse model, AR expression was assessed in different tissues. AR mRNA levels in testis, heart, kidney, brain, fat, liver and quadriceps muscle were not different between control and ocl-ARKO mice (Fig. 1B). However, as shown in Fig. 1A, AR mRNA was very low in osteoclast-enriched cells, and decreased during *in vitro* differentiation of hematopoietic cells toward osteoclasts. No significant differences in AR expression could be demonstrated at the mRNA level (Fig. 1A). Cre mediated excision was detected with PCR on genomic DNA in ocl-ARKO and not in controls (Fig. 1C), although a large $AR^{flox/Y}$ band (930 bp) was still visible. *Ctsk* expression was very high in the osteoclast-enriched cultures, with no significant difference between genotypes (data not shown).

Deletion of the AR on osteoclasts had neither an effect on trabecular/cortical bone nor on serum bone turnover markers (Table 1), as determined at 12 and 32 weeks of age. In consistence with the lack of bone phenotype, osteoclast surface (Oc.S/BS) remained unaltered in the femur of ocl-ARKO mice compared to controls regardless of age, although 32-week-old animals showed higher values (Table 1).

The response in trabecular bone volume following ORX and T replacement was similar in ocl-ARKO and controls (Fig. 2). Androgen dosage was slightly supraphysiological, since the seminal vesicle weight corrected for body weight was significantly higher in the T replacement groups compared to sham mice (13.1 mg/g BW ± 0.5 *vs.* 9.0 mg/g ± 0.4). The weight of the seminal vesicles after ORX was very low (0.2 mg/g BW ± 0.4).

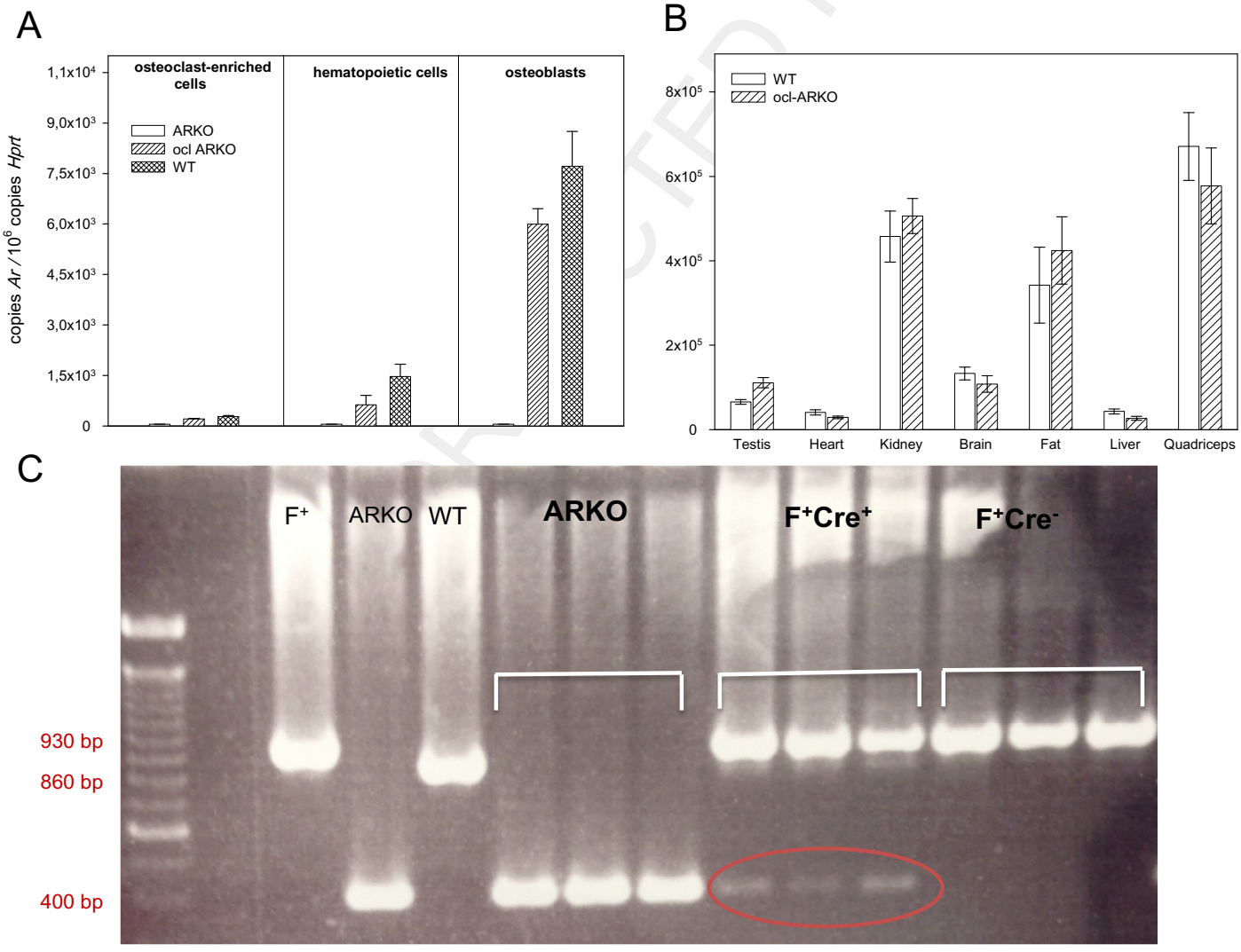

Fig. 1. Generation and validation of ocl-ARKO mice. (A) Quantitative real-time PCR analysis of AR mRNA in osteoclast-enriched, BM-derived hematopoietic and calvarial osteoblast cell cultures of ARKO, ocl-ARKO and control mice. Values are expressed as the mean of three independent experiments (n = 3 mice/group in each experiment) ± SEM. (B) Quantitative real-time PCR analysis of AR in different tissues of control and ocl-ARKO mice (n = 8–10). (C) PCR analysis of extracted DNA from osteoclast-enriched cultures derived from ARKO, ocl-ARKO ($AR^{flox/Y}$Ctsk-$Cre^{+/-}$) and flox control ($AR^{flox/Y}$Ctsk-$Cre^{-/-}$) mice. The 400 bp PCR fragment is detected in ocl-ARKO (circled in red), indicating Cre-mediated excision. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

Table 1
μCT and histomorphometric analysis of femur of 12- and 32-week-old ocl-ARKO and control mice as well as bone turnover markers.

	12 w		32 w	
	Control	ocl-ARKO	Control	ocl-ARKO
Femur length (mm)	15.3 ± 1.26	15.5 ± 1.6	16.1 ± 2.38	15.8 ± 1.15
BV/TV (%)	13 ± 5.38	14 ± 5.1	12 ± 6	13 ± 4.2
Tb.Th (μm)	40 ± 10.2[a]	38 ± 8.1[b]	130 ± 32.1	128 ± 36.6
Tb.N (1/mm)	3.2 ± 2.7[a]	3.3 ± 2.4[b]	2.0 ± 2.1	1.9 ± 1.5
Tb.Sp (μm)	200 ± 28.2[a]	203 ± 30.6[b]	250 ± 33.9	245 ± 38.1
Ps.Pm (mm)	5.4 ± 1.71	5.2 ± 1.02	5.3 ± 1.29	5.3 ± 1.53
Ec.Pm (mm)	3.3 ± 0.14[a]	3.2 ± 0.28[b]	3.82 ± 0.14	3.67 ± 0.16
Ct.Ar (mm^2)	0.952 ± 0.07	0.924 ± 0.14	0.907 ± 0.11	0.894 ± 0.09
Me.Ar (mm^2)	1.52 ± 0.16[a]	1.47 ± 0.14[b]	1.14 ± 0.17	1.15 ± 0.156
Ct.Th (μm)	187 ± 12.03	183 ± 15.72	185 ± 11.37	190 ± 12.99
Oc. S/BS (%)	3.1 ± 0.5[a]	3.4 ± 1.6	5.6 ± 2	4.7 ± 1.59
Osteocalcin (ng/mL)	80 ± 21.9	81 ± 12.9	ND	ND
TRACP5b (ng/mL)	11 ± 10.2	12 ± 13.2	ND	ND

Bone volume/tissue volume (BV/TV); trabecular thickness (Tb.Th); trabecular number (Tb.N); trabecular separation (Tb.Sp); periosteal perimeter (Ps.Pm); endocortical perimeter (Ec.Pm); cortical area (Ct.Ar); medullar area (Me.Ar); cortical thickness (Ct.Th); osteoclast surface/bone surface (Oc.S/BS). Values are expressed as means (n = 4–10 mice/group) ± SD.

[a] $p < 0.05$ *vs.* control 32 weeks.

[b] $p < 0.05$ *vs.* ocl-ARKO 32 weeks.

3.2. AR gene expression in different bone marrow cell populations

Different cell populations were derived from the BM of ARKO and corresponding WT mice. For this purpose, tibiae were flushed and BM-derived cells were plated after Ficoll purification. After 24 h in culture, the non-adherent cells (called here hematopoietic cells) were cultured in the presence of RANKL and M-CSF to form the osteoclast-enriched cells. The term "osteoclast-enriched cells" is used since it cannot be excluded that some stromal cells are still present. To circumvent this potential stromal contamination, mature osteoclasts were also obtained from spleen precursors. BMSCs were obtained by further culturing the adherent cells (Fig. 3A).

First, AR expression was assessed in different bone fractions from WT mice: whole tibia, freshly-isolated BM, hematopoietic cells, osteoclast-enriched cells (after culturing with addition of M-CSF and RANKL) and stromal cells (Fig. 3A and B). AR mRNA expression was the highest in stromal cells, very low in osteoclast-enriched cells and absent in osteoclasts derived from spleen precursors (Ct values equal to that of global ARKO).

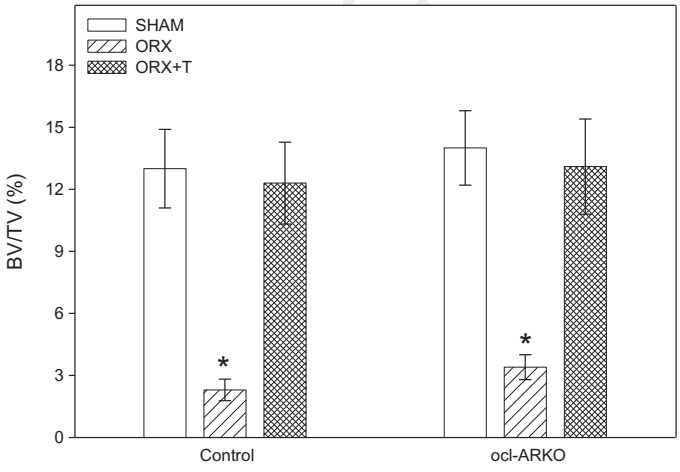

Fig. 2. Trabecular bone volume/tissue volume (BV/TV) (%) assessed by micro-CT in the femur in control and ocl-ARKO mice after ORX and ORX+T. Values are expressed as mean (n = 10–15 mice/group) ± SEM. *$p < 0.05$ *vs.* rest of the experimental groups.

3.3. Osteoclastogenesis and osteoclast activity in ARKO and WT mice

ARKO mice showed higher TRAP-positive osteoclast surface compared to WT (Fig. 4A). In contrast, when BM cells were cultured *in vitro* in the presence of RANKL and M-CSF to induce osteoclastogenesis, the number of osteoclasts generated as well as their activity was the same in ARKO and WT mice (Fig. 4B and C).

Next, we performed *in vitro* co-culture experiments of calvarial osteoblasts from ARKO and WT mice (which provide M-CSF and RANKL for osteoclast generation when treated with $1,25(OH)_2D_3$), with BM-derived cells from ARKO and WT mice. Osteoclast generation and resorption activity were greater when both cell types in the co-culture were from ARKO mice (Fig. 5A and B, left part). However, we found that this effect could be attributed mainly to the BM-derived cell fraction and not to the osteoblasts (Fig. 5A and B, right part).

3.4. Rankl, Opg, inflammatory cytokines and Runx2 expression in osteoblasts and BMSCs from WT and ARKO mice

Finally, we examined the expression of key regulator genes involved in osteoclastogenesis and osteoblastogenesis in osteoblasts and BMSCs from WT and ARKO mice. As shown in Fig. 6, *Rankl* mRNA was not different in calvarial osteoblasts between ARKO and WT. As expected, *Rankl* expression increased in osteoblasts from both ARKO and WT after stimulation with $1,25(OH)_2D_3$. In BMSCs, the *Rankl* expression was very low and, although it was significantly higher in ARKO BMSCs compared to WT, $1,25(OH)_2D_3$ treatment increased the expression in both genotypes and abolished any difference between them (Fig. 6). *Opg* expression was not different between calvarial osteoblasts from ARKO and WT and decreased significantly after $1,25(OH)_2D_3$ but only in WT osteoblasts (Fig. 6). *Opg* mRNA was significantly higher in ARKO compared to WT BMSCs, but only after treatment with $1,25(OH)_2D_3$ (Fig. 6). Consequently, the *Rankl/Opg* ratios were similar between WT and ARKO osteoblasts, with no evidence of increased sensitivity to $1,25(OH)_2D_3$ stimulation (Fig. 6). The *Rankl/Opg* ratio was upregulated to a greater extent by $1,25(OH)_2D_3$ stimulation in WT compared to ARKO BMSCs (Fig. 6), which was mainly due to lower OPG gene expression in WT stromal cells (Fig. 6). All in all, neither osteoblasts nor BMSCs from ARKO showed an increased *Rankl* or decreased *Opg* expression, which could have been expected given the increased osteoclast surface in ARKO mice *in vivo*.

We also determined the transcript levels of the main osteoclastogenic cytokines, namely TNF-α, IL-1α, IL-1β and IL-6. Overall, the expression of the pro-inflammatory cytokines was indistinguishable between ARKO and WT BMSCs and osteoblasts, regardless of $1,25(OH)_2D_3$ treatment (data not shown). The effects of $1,25(OH)_2D_3$ stimulation on mRNA levels were restricted to IL-1α in stromal cells, while the expression of the cytokine in osteoblasts was below the detection limits (Fig. 6). As expected, the expression of *Runx2* (an osteoprogenitor marker) was much higher in osteoblasts compared to BMSCs (Fig. 6). There was no difference in *Runx2* expression between ARKO or WT osteoblasts, regardless of the presence of absence of $1,25(OH)_2D_3$. However, *Runx2* expression was significantly higher in ARKO BMSCs, both with and without $1.25(OH)_2D_3$ stimulation (Fig. 6).

4. Discussion

The major finding of this study is that AR expression in mouse osteoclasts is extremely low/absent and dispensable for the androgen-mediated inhibition of bone resorption in male mice. This is in line with earlier studies in ORX mice with defective osteoblastogenesis, which showed that up-regulation of osteoblastogenesis appears

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

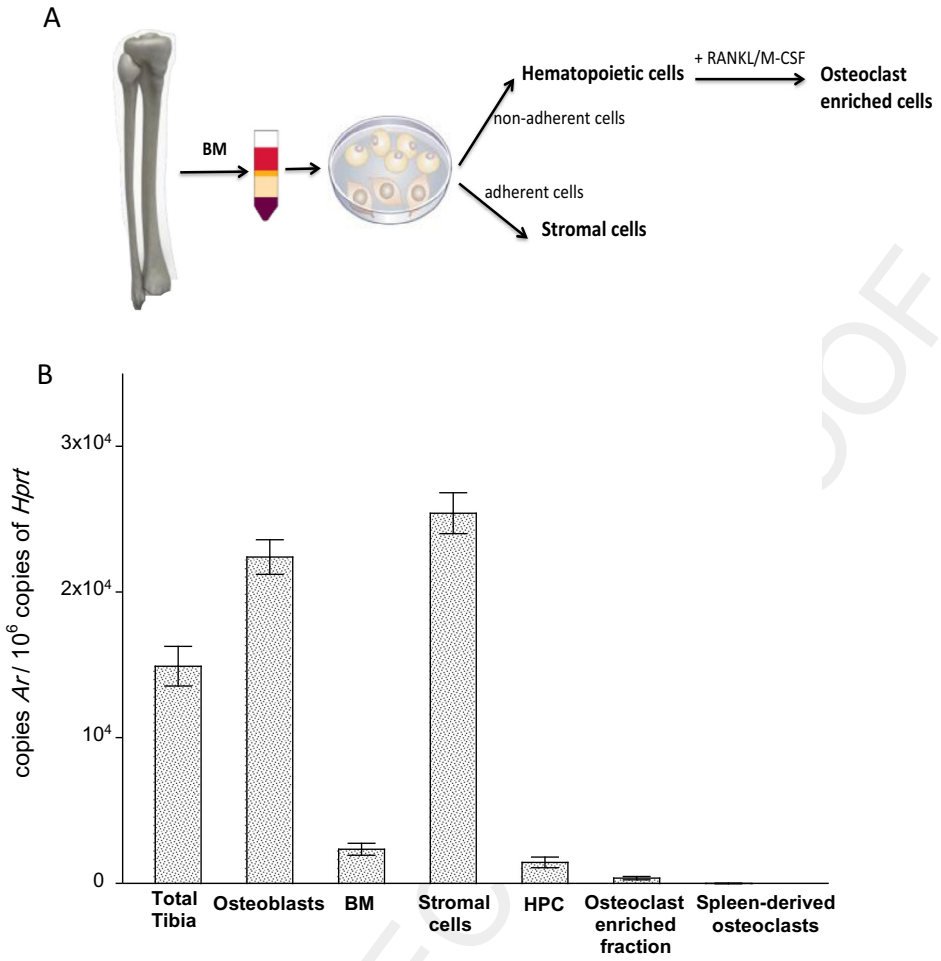

Fig. 3. (A) Overview of bone fractions employed in this study. Detailed protocols are given in Section 2. (B) AR mRNA expression levels relative to hypoxanthine–guanine phosphoribosyltransferase (HPRT) in total tibia, calvarial osteoblasts, bone marrow (BM), stromal cells, hematopoietic cells (HPC), osteoclast-enriched fraction and osteoclasts derived from spleen precursors. Values are the mean of three independent experiments (n = 3 mice/group in each experiment) ± SEM.

essential for the subsequent rise of osteoclastogenesis, arguing again against a direct suppressive role of AR on osteoclasts or their precursors (Weinstein et al., 1997).

Nevertheless, AR and ERα in osteoclasts have been suggested to be involved in the well-documented anti-resorptive actions of estrogens and androgens on bone. AR expression has been detected by IHC and *in situ*-hybridization in avian and mouse osteoclasts *in vitro* as well as in rat osteoclasts *in vivo* (Mizuno et al., 1994; Pederson et al., 1999; Turner et al., 2008; van der Eerden et al., 2002). Furthermore, androgens have been reported to inhibit osteoclast generation and activity *in vitro* according to some (Chen et al., 2001; Huber et al., 2001; Pederson et al., 1999) but not all studies (Caputo et al., 1976; Tobias and Chambers, 1991). *In vivo*, ERα has been shown to inhibit bone resorption via direct actions in osteoclasts in female but not in male mice (Martin-Millan et al., 2010; Nakamura et al., 2007). These results opened the possibility that the AR could have a similar role in osteoclasts in male mice, as suggested by the work of Nakamura et al. in 2004, in which the Cre expression was also controlled by the cathepsin K (Ctsk) promoter (Nakamura et al., 2004).

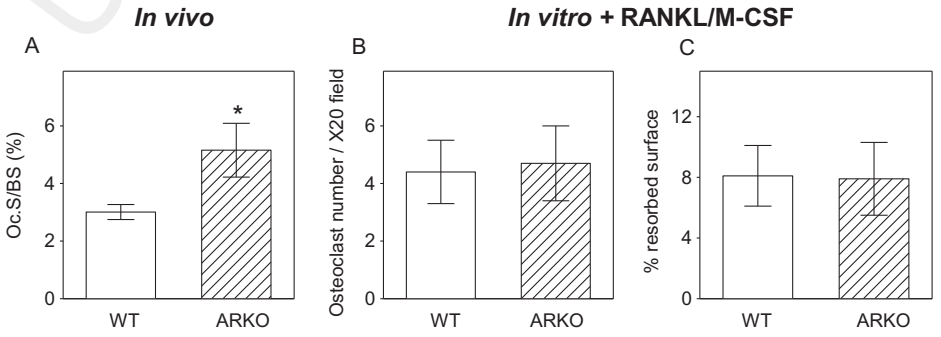

Fig. 4. (A) Osteoclast surface/bone surface (Oc.S/BS) measured *in vivo* on tibia after TRAP staining, from 12-week-old male WT and ARKO mice. (B) Osteoclast number X20 field and (C) % resorbed surface/total surface by osteoclasts.

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

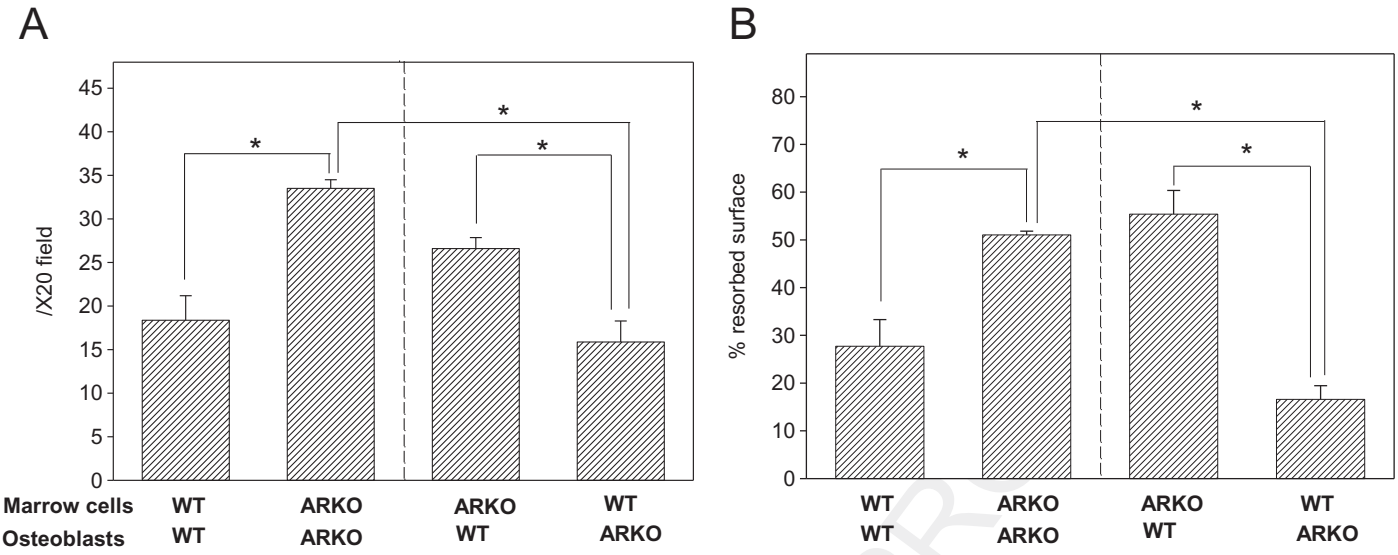

Fig. 5. (A) Osteoclastogenesis in co-cultures of bone marrow (BM) cells and osteoblasts. TRAP-positive multinucleated osteoclast numbers were counted after 8-day co-culture of BM cells and osteoblasts from male ARKO and WT mice in the presence of 10 nM $1,25(OH)_2D_3$. (B) Pit area resorbed by osteoclasts cultured on Osteo Assay Surface. $^*p < 0.05$.

To the best of our knowledge, AR expression in murine osteoclasts as assessed by qPCR has only been determined in two studies thus far (Turner et al., 2008; Ucer et al., 2015). AR mRNA levels in the *in vitro* generated osteoclasts were, however, extremely low and the possibility of contamination with AR from stromal cells could not be excluded. Accordingly, in our conditions the AR expression was also very low in osteoclast-enriched cells compared to osteoblasts or BM cells. Moreover, here, we generated mature osteoclasts from spleen precursors in order to circumvent the potential stromal contamination in the osteoclast-enriched fraction. Interestingly, AR mRNA expression was absent in these cultures, being the CT values equal to that of global ARKO (background noise). Therefore, these new findings suggest that murine osteoclasts do not represent a direct cellular target for androgenic action. Nevertheless, we tried to generate an osteoclast-specific ARKO using Ctsk-Cre mice (Chiu et al., 2004). The Ctsk-Cre mice have been previously used to successfully knockout the calcitonin receptor specifically in osteoclasts (Turner et al., 2011). In contrast to global ARKO mice (Callewaert et al., 2009; Kawano et al., 2003) and the earlier reported abstract of osteoclast-specific ARKO mice (Nakamura et al., 2004), our ocl-ARKO mice did not exhibit osteopenia nor changes in osteoclast surface and responded normally to ORX as well as androgen replacement *in vivo*. However, our findings are in agreement with a recent study by Ucer et al. using an ocl-ARKO mouse model driven by the LysM promoter (Ucer et al., 2015). The authors failed to demonstrate a bone phenotype in these animals despite that in their ocl-ARKO the AR was already deleted in myeloid precursors. Therefore, the study discussed earlier rules out the possibility that the osteoclast stage was a determinant factor for the observed negative results here.

In contrast to ocl-ARKO mice, tibiae of ubiquitous ARKO mice displayed increased osteoclast counts Therefore, the role of the AR was further explored using cell cultures from these animals and their WT littermates. In accordance with earlier studies, *in vitro* osteoclastogenesis as well as resorption activity was not different in the global ARKO *vs.* WT in the presence of RANKL and M-CSF, which again argues against a direct role for AR in osteoclasts as well as in osteoclast precursor cells (Kawano et al., 2003). Thus, in the next set of experiments, we co-cultured osteoblasts and BMSCs in order to test if the AR might control osteoclast activity indirectly by acting on these bone cell lineages. Also in agreement with earlier data, both osteoclast generation and activity were suppressed in the presence of AR in osteoblasts and BM cell co-cultures (Kawano et al., 2003). In contrast with these earlier findings (Kawano et al., 2003) however, co-culturing WT osteoblasts with ARKO BM cells and *vice versa* showed that BM cells and not osteoblasts mediate the increased osteoclast generation and activity in ARKO mice (Fig. 5). Because the BM cell fraction consists of hematopoietic-lineage cells (pre-osteoclasts) and BMSCs, and no direct effect via hematopoietic cells could be demonstrated (Fig. 4), we hypothesize that BMSCs are more important for the effects of AR on the regulation of bone resorption than currently realized. Similarly, a recent study reported that AR in stromal cells contributes to the effects of androgens on muscle (Ipulan et al., 2014). However, care must be taken not to overinterpret results from *in vitro* studies, and the contribution of mesenchymal stromal cells to AR effects on bone requires confirmation in future conditional mouse deletion studies.

Both osteoblasts and osteoblast progenitor cells produce RANKL and OPG which are key factors for osteoclast formation and activity. Contrary to earlier findings however, we found no evidence that AR suppresses bone resorption by regulating the RANKL/OPG pathway (Kawano et al., 2003). On the contrary, we found that *Opg* mRNA expression was increased in ARKO BMSCs, in line with other previous studies (Hofbauer et al., 2002). Soluble RANKL in BM may still increase following androgen deficiency via indirect mechanisms, such as increased release by matrix metalloproteinases (Proell et al., 2009).

Interestingly, the expression of *Runx2*, which is a marker of commitment to the osteoblast lineage, was also significantly higher in ARKO compared to WT BMSCs (Fig. 6H). Although suboptimal RUNX2 activity leads to compromised osteoblastogenesis and insufficient bone formation, supra-optimal activity also leads to an exaggerated osteoclastogenesis and high-turnover osteopenia (Liu et al., 2001). Therefore, we hypothesize that the high-turnover osteopenia in ARKO mice might be due to unrestrained osteoblastogenesis because the AR fails to suppress osteoblast commitment of BMSCs.

In conclusion, the presence of AR in osteoclasts is doubtful, and does not explain the suppressive action of AR on osteoclastogenesis and activity. Suppression of BMSC commitment to the osteoblast lineage may represent an alternative indirect mechanism for the antiresorptive action of AR which requires further confirmation.

Acknowledgments

The authors thank E. Van Herck, K. Moermans, R. Van Looveren, R. Bollen and S. Torrekens for their excellent technical assistance.

Please cite this article in press as: Mieke Sinnesael, et al., The androgen receptor has no direct antiresorptive actions in mouse osteoclasts, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.030

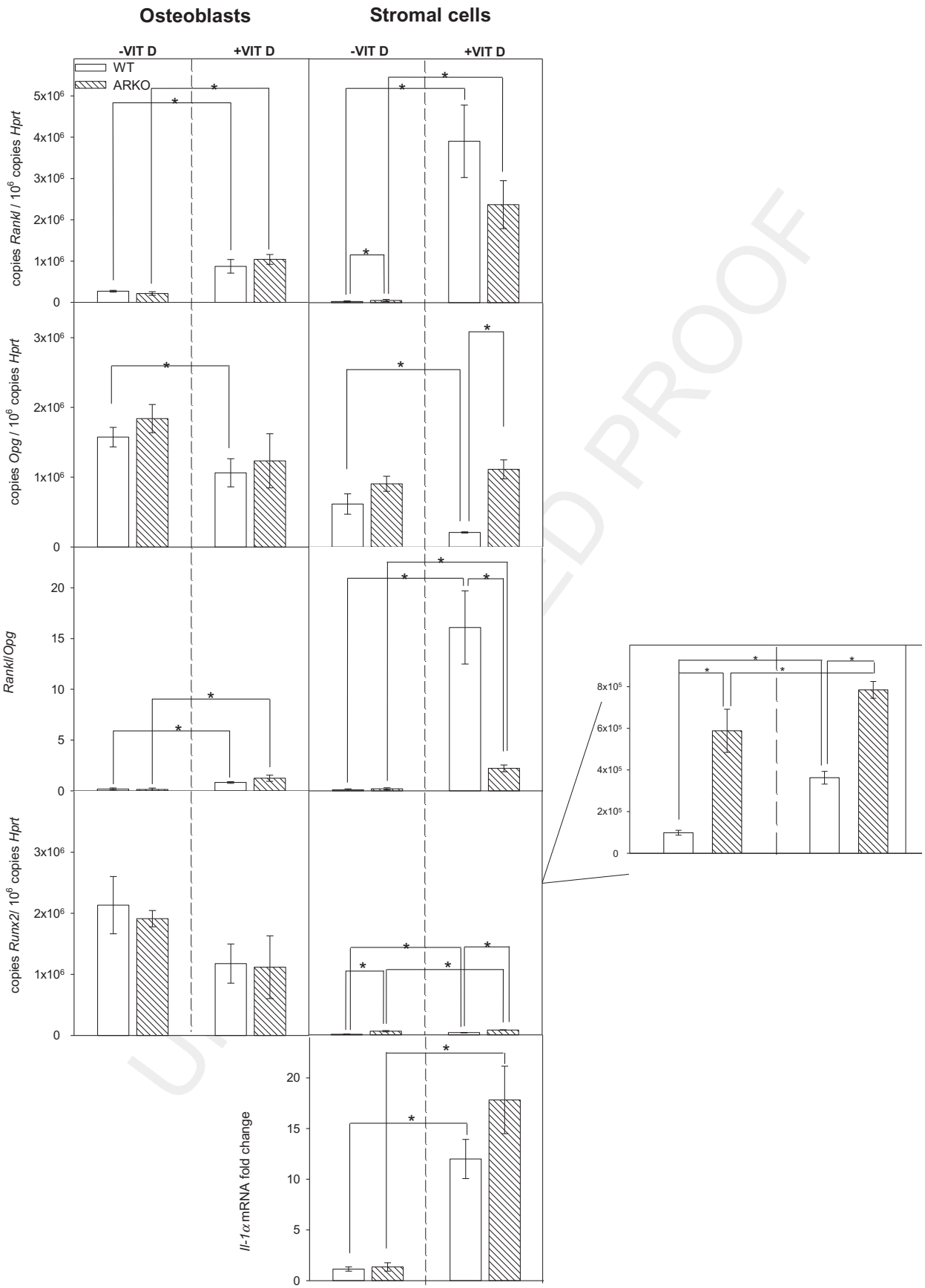

Fig. 6. Effect of 1.25$(OH)_2D_3$ treatment on mRNA levels of *Rankl*, *Opg*, *Il-1α*, *Runx2* and Rankl/Opg ratio in calvarial osteoblasts and BMSCs derived from WT and ARKO mice. Values are expressed as the mean of three independent experiments (n = 3 mice/group in each experiment) ± SEM. $^*p < 0.05$.

We would also like to thank the assistance with spleen cell cultures provided by Dr. Carlier and Dr. VanderElst (Center for Molecular and Vascular Biology, Leuven, Belgium).

This study was funded by grant OT/09/035 and GOA/15/017 from KU Leuven and grant number G.0858.11 from the Research Foundation Flanders (FWO). M.R.L. and V.D. are PhD fellows of the Research Foundation Flanders (FWO). D.V. is senior investigator of the University Hospitals Leuven Clinical Research Fund.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.04.030.

References

Arai, F., Miyamoto, T., Ohneda, O., Inada, T., Sudo, T., Brasel, K., et al., 1999. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J. Exp. Med. 190, 1741–1754.

Bellido, T., Jilka, R.L., Boyce, B.F., Girasole, G., Broxmeyer, H., Dalrymple, S.A., et al., 1995. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. J. Clin. Invest. 95, 2886–2895.

Bradley, E.W., Oursle, M.J., 2008. Osteoclast culture and resorption assays. In: Osteoporosis Methods and Protocols, vol. 455. Humana Press, Totowa, New Jersey, pp. 19–35.

Callewaert, F., Venken, K., Ophoff, J., De Gendt, K., Torcasio, A., van Lenthe, G.H., et al., 2009. Differential regulation of bone and body composition in male mice with combined inactivation of androgen and estrogen receptor-alpha. FASEB J. 23, 232–240.

Caputo, C.B., Meadows, D., Raisz, L.G., 1976. Failure of estrogens and androgens to inhibit bone resorption in tissue culture. Endocrinology 98, 1065–1068.

Chen, Q., Kaji, H., Sugimoto, T., Chihara, K., 2001. Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor. FEBS Lett. 491, 91–93.

Chiang, C., Chiu, M., Moore, A.J., Anderson, P.H., Ghasem-Zadeh, A., McManus, J.F., et al., 2009. Mineralization and bone resorption are regulated by the androgen receptor in male mice. J. Bone Miner. Res. 24, 621–631.

Chiu, W.S.M., McManus, J.F., Notini, A.J., Cassady, A.I., Zajac, J.D., Davey, R.A., 2004. Transgenic mice that express Cre recombinase in osteoclasts. Genesis 39, 178–185.

Daci, E., Verstuyf, A., Moermans, K., Bouillon, R., Carmeliet, G., 2000. Mice lacking the plasminogen activator inhibitor 1 are protected from trabecular bone loss induced by estrogen deficiency. J. Bone Miner. Res. 15, 1510–1516.

De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., et al., 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. U.S.A. 101, 1327–1332.

Falahati-Nini, A., Riggs, B.L., Atkinson, E.J., O'Fallon, W.M., Eastell, R., Khosla, S., 2000. Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. J. Clin. Invest. 106, 1553–1560.

Hofbauer, L.C., Hicok, K.C., Chen, D., Khosla, S., 2002. Regulation of osteoprotegerin production by androgens and anti-androgens in human osteoblastic lineage cells. Eur. J. Endocrinol. 147, 269–273.

Huber, D.M., Bendixen, A.C., Pathrose, P., Srivastava, S., Dienger, K.M., Shevde, N.K., et al., 2001. Androgens suppress osteoclast formation induced by RANKL and macrophage-colony stimulating factor. Endocrinology 142, 3800–3808.

Idan, A., Griffiths, K.A., Harwood, D.T., Seibel, M.J., Turner, L., Conway, A.J., et al., 2010. Long-term effects of dihydrotestosterone treatment on prostate growth in healthy, middle-aged men without prostate disease: a randomized, placebo-controlled trial. Ann. Intern. Med. 153, 621–632.

Imai, Y., Youn, M.Y., Inoue, K., Takada, I., Kouzmenko, A., Kato, S., 2013. Nuclear receptors in bone physiology and diseases. Physiol. Rev. 93, 481–523.

Ipulan, L.A., Suzuki, K., Sakamoto, Y., Murashima, A., Imai, Y., Omori, A., et al., 2014. Nonmyocytic androgen receptor regulates the sexually dimorphic development of the embryonic bulbocavernosus muscle. Endocrinology 155, 2467–2479.

Kawano, H., Sato, T., Yamada, T., Matsumoto, T., Sekine, K., Watanabe, T., et al., 2003. Suppressive function of androgen receptor in bone resorption. Proc. Natl. Acad. Sci. U.S.A. 100, 9416–9421.

Khosla, S., Amin, S., Orwoll, E., 2008. Osteoporosis in men. Endocr. Rev. 29, 441–464.

Komori, T., 2011. Signaling networks in RUNX2-dependent bone development. J. Cell. Biochem. 112, 750–755.

Laurent, M.R., Gielen, E., Vanderschueren, D., 2015. Estrogens, the be-all and end-all of male hypogonadal bone loss? Osteoporos. Int. 26, 29–33.

Liu, W., Toyosawa, S., Furuichi, T., Kanatani, N., Yoshida, C., Liu, Y., et al., 2001. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J. Cell Biol. 155, 157–166.

Manolagas, S.C., O'Brien, C.A., Almeida, M., 2013. The role of estrogen and androgen receptors in bone health and disease. Nat. Rev. Endocrinol. 9, 699–712.

Martin-Millan, M., Almeida, M., Ambrogini, E., Han, L., Zhao, H., Weinstein, R.S., et al., 2010. The estrogen receptor-alpha in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. Mol. Endocrinol. 24, 323–334.

Masuyama, R., Stockmans, I., Torrekens, S., Van Looveren, R., Maes, C., Carmeliet, P., et al., 2006. Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. J. Clin. Invest. 116, 3150–3159.

Määttä, J.A., Büki, K.G., Ivaska, K.K., Nieminen-Pihala, V., Elo, T.D., Kähkönen, T., et al., 2013. Inactivation of the androgen receptor in bone-forming cells leads to trabecular bone loss in adult female mice. Bonekey Rep. 2, 440.

Mizuno, Y., Hosoi, T., Inoue, S., Ikegami, A., Kaneki, M., Akedo, Y., et al., 1994. Immunocytochemical identification of androgen receptor in mouse osteoclast-like multinucleated cells. Calcif. Tissue Int. 54, 325–326.

Nakamura, T., Watanabe, T., Nakamichi, Y., Fukuda, T., Matsumoto, T., Yoshimura, K., et al., 2004. Genetic evidence of androgen receptor function in osteoclasts: generation and characterization of osteoclast-specific androgen receptor knockout mice. J. Bone Miner. Res. 19, S3.

Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., et al., 2007. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130, 811–823.

Notini, A.J., McManus, J.F., Moore, A., Bouxsein, M., Jimenez, M., Chiu, W.S., et al., 2007. Osteoblast deletion of exon 3 of the androgen receptor gene results in trabecular bone loss in adult male mice. J. Bone Miner. Res. 22, 347–356.

Pederson, L., Kremer, M., Judd, J., Pascoe, D., Spelsberg, T.C., Riggs, B.L., et al., 1999. Androgens regulate bone resorption activity of isolated osteoclasts in vitro. Proc. Natl. Acad. Sci. U.S.A. 96, 505–510.

Proell, V., Xu, H., Schuler, C., Weber, K., Hofbauer, L.C., Erben, R.G., 2009. Orchiectomy upregulates free soluble RANKL in bone marrow of aged rats. Bone 45, 677–681.

Seeman, E., 2003. Periosteal bone formation – a neglected determinant of bone strength. NEJM 349, 320–323.

Sims, N.A., Martin, T.J., 2014. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. Bonekey Rep. 3, 481.

Sinnesael, M., Claessens, F., Laurent, M., Dubois, V., Boonen, S., Deboel, L., et al., 2012. Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity: evidence from targeted AR disruption in mouse osteocytes. J. Bone Miner. Res. 27, 2535–2543.

Tobias, J.H., Chambers, T.J., 1991. The Effect of Sex Hormones on Bone Resorption by Rat Osteoclasts, vol. 124. Acta Endocrinologica, Copenhagen, pp. 121–127.

Turner, A.G., Notini, A.J., Chiu, W.S.M., Hodge, J., Nicholson, G., Zajac, J.D., et al., 2008. Androgen receptor expression and function in osteoclasts. Open Physiol. J. 1, 28–33.

Turner, A.G., Tjahyono, F., Chiu, W.S., Skinner, J., Sawyer, R., Moore, A.J., et al., 2011. The role of the calcitonin receptor in protecting against induced hypercalcemia is mediated via its actions in osteoclasts to inhibit bone resorption. Bone 48, 354–361.

Ucer, S., Iyer, S., Bartell, S.M., Martin-Millan, M., Han, L., Kim, H.N., et al., 2015. The effects of androgens on murine cortical bone do not require AR or ERα signaling in osteoblasts and osteoclasts. J. Bone Miner. Res. doi:10.1002/jbmr.2485; Epub ahead of print.

van der Eerden, B.C., van Til, N.P., Brinkmann, A.O., Lowik, C.W., Wit, J.M., Karperien, M., 2002. Gender differences in expression of androgen receptor in tibial growth plate and metaphyseal bone of the rat. Bone 30, 891–896.

Vanderschueren, D., Vandenput, L., Boonen, S., Van Herck, E., Swinnen, J.V., Bouillon, R., 2000. An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. Endocrinology 141, 1642–1647.

Vanderschueren, D., Laurent, M.R., Claessens, F., Gielen, E., Lagerquist, M.K., Vandenput, L., et al., 2014. Sex steroid actions in male bone. Endocr. Rev. er20141024.

Venken, K., De Gendt, K., Boonen, S., Ophoff, J., Bouillon, R., Swinnen, J.V., et al., 2006. Relative impact of androgen and estrogen receptor activation in the effects of androgens on trabecular and cortical bone in growing male mice: a study in the androgen receptor knockout mouse model. J. Bone Miner. Res. 21, 576–585.

Verhaeghe, J., Van Herck, E., Van Bree, R., Van Assche, F.A., Bouillon, R., 1989. Osteocalcin during the reproductive cycle in normal and diabetic rats. J. Endocrinol. 120, 143–151.

Verlinden, L., Kriebitzsch, C., Beullens, I., Tan, B.K., Carmeliet, G., Verstuyf, A., 2013. Nrp2 deficiency leads to trabecular bone loss and is accompanied by enhanced osteoclast and reduced osteoblast numbers. Bone 55, 465–475.

Weinstein, R.S., Jilka, R.L., Parfitt, A.M., Manolagas, S.C., 1997. The effects of androgen deficiency on murine bone remodeling and bone mineral density are mediated via cells of the osteoblastic lineage. Endocrinology 138, 4013–4021.

Xiong, J., Onal, M., Jilka, R.L., Weinstein, R.S., Manolagas, S.C., O'Brien, C.A., 2011. Matrix-embedded cells control osteoclast formation. Nat. Med. 17, 1235–1241.

Uncited reference

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., et al., 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89, 755–764.